

# Neuronal Regulation of the Spatial Patterning of Neurogenesis

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## SUMMARY

Precise regulation of neurogenesis is achieved in specific regions of the vertebrate nervous system by formation of distinct neurogenic and nonneurogenic zones. We have investigated how neurogenesis becomes confined to zones adjacent to rhombomere boundaries in the zebrafish hindbrain. The nonneurogenic zone at segment centers comprises a distinct progenitor population that expresses *fibroblast growth factor (fgfr) 2*, *erm*, *sox9b*, and the retinoic acid degrading enzyme, *cyp26b1*. FGF receptor activation upregulates expression of these genes and inhibits neurogenesis in segment centers. Cyp26 activity is a key effector inhibiting neuronal differentiation, suggesting antagonistic interactions with retinoid signaling. We identify the critical FGF ligand, *fgf20a*, which is expressed by specific neurons located in the mantle region at the center of segments, adjacent to the nonneurogenic zone. *Fgf20a* mutants have ectopic neurogenesis and lack the segment center progenitor population. Our findings reveal how signaling from neurons induces formation of a nonneurogenic zone of neural progenitors.

## INTRODUCTION

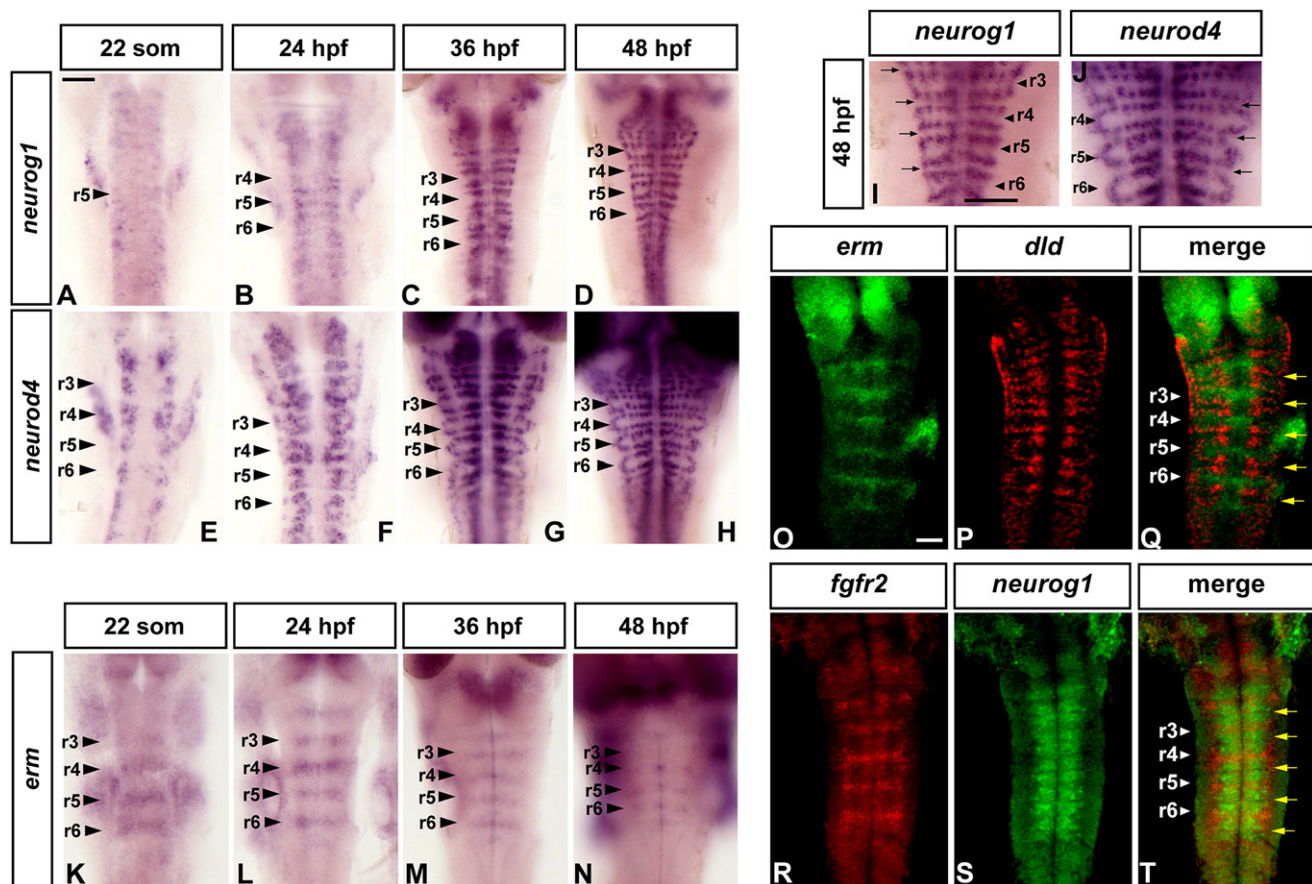
During development of the vertebrate central nervous system, progenitor cells that comprise the neural epithelium differentiate to form a wide variety of neuronal and glial cell types at appropriate locations and in correct numbers. The specific cell types formed are regulated spatially by signals that underlie subdivision of the neural epithelium into domains with distinct identity, or that specify distinct subtypes within these regions. Different neuronal and glial cell types are born at different times, with switches in the differentiation of a progenitor population to generate, for example, initially neurons, and subsequently glial cells (Guillemot, 2007). This generation of differentiated cells over a prolonged period necessitates that sufficient neural progenitor cells are maintained throughout development. The differentiation of the correct number of neurons and maintenance of progenitors are regulated by extrinsic and intrinsic factors that promote or inhibit neurogenesis, and that regulate

the proliferation of neural epithelial cells (Bertrand et al., 2002; Diez del Corral and Storey, 2001).

Neurogenesis is initiated by the upregulation of proneural transcription factors that trigger a downstream cascade of genes that control further steps of neuronal differentiation (Bertrand et al., 2002). The amount of neurogenesis is limited by a number of factors that inhibit the upregulation or the activity of proneural proteins. Some inhibitory factors are widely expressed in the neural epithelium; for example, members of the SoxB1 (Sox1, Sox2, Sox3) family of proteins that have a major role in maintenance of neural progenitor cells (Bylund et al., 2003; Graham et al., 2003). Another crucial regulation of neurogenesis occurs through local cell-cell signaling in the process of lateral inhibition. Proneural genes upregulate the expression of Notch ligands, which, by activating the Notch receptor in adjacent cells, upregulate Hes/Her transcriptional repressors that inhibit neurogenesis (Fisher and Caudy, 1998). This Notch-mediated lateral inhibition ensures that progenitor cells are maintained within regions in which neurogenesis is occurring.

A further mechanism to regulate neurogenesis occurs in some regions of the neural epithelium in which there is a large-scale spatial organization of neurogenic and nonneurogenic zones. Such patterning has been found to be generated by spatially restricted inhibitory mechanisms that confine neurogenesis to specific regions (Bally-Cuif and Hammerschmidt, 2003; Diez del Corral and Storey, 2001). For example, neurogenesis does not occur at the midbrain-hindbrain boundary, or in interpro-neuronal domains along the dorsoventral axis of the spinal cord, due to Notch-independent expression of specific *Hes/Her* genes (Bae et al., 2005; Geling et al., 2003, 2004) or to the expression of members of the *Zic* gene family (Brewster et al., 1998).

An important question is the identity of extrinsic signaling factors that regulate the promotion or inhibition of neurogenesis. A number of signals have been implicated in the control of neurogenesis, including retinoic acid (RA), Wnt, BMP, Shh, and fibroblast growth factor (FGF) family members (Dono, 2003; Ford-Perriss et al., 2001; Maden, 2007; Michaelidis and Lie, 2008; Pozniak and Pleasure, 2006; Sharpe and Goldstone, 1997, 2000; Shi et al., 2008; Wu et al., 2003). In some cases, these signals are expressed in specific centers within the neural epithelium or adjacent tissues, and are involved in a coordinated regulation of neurogenesis and patterning along the anterior-posterior or dorsoventral axis. These factors can act in a cooperative or antagonistic manner; for example, the promotion of neurogenesis by RA being opposed by FGF signaling in the caudal spinal cord (Diez del Corral et al., 2003; Diez del Corral and Storey, 2004). However, for many of these signals, their



**Figure 1. FGF Signaling Is Restricted to Nonneurogenic Regions in the Zebrafish Hindbrain**

Dorsal views of flat mounted embryos, anterior to the top, at the indicated stages. Following in situ hybridization, embryos of the same batch were developed for the same amount of time. Arrowheads indicate segment centers; arrows point at hindbrain boundaries. Scale bars, 50  $\mu$ m. (A–H) Time course of *neurog1* (A–D) and *neurod4* (E–H) expression from 22 somites to 48 hr. (I and J) Higher-power views showing the spatial restriction of neurogenesis marked by *neurog1* (same embryo as [D]) and *neurod4* (J), same embryo as [H]). (K–N) *erm* expression. (O–T) Double fluorescent in situ hybridization using probes for *erm* and *dld* (O–Q), and *fgfr2* and *neurog1* (R–T). Images shown are a merge of confocal stacks through the hindbrain at 36 hpf.

relationship with neurogenesis is complex and context dependent; for example, FGF signaling has been implicated in the self-renewal of neural stem cells (Gage et al., 1995; Gritti et al., 1996), and in the promotion or inhibition of neurogenesis (Borello et al., 2008; Ford-Perriss et al., 2001; Hardcastle et al., 2000; Topp et al., 2008).

Previous studies have shown that, in the zebrafish hindbrain, neurogenesis becomes confined to zones that flank segment boundaries, and does not occur in the central region of each segment (Amoyel et al., 2005; Cheng et al., 2004). We set out to investigate how this stereotyped pattern of neurogenic and nonneurogenic progenitor zones is established. We report that FGF receptor (FGFR) activation occurs in the center of segments, where it upregulates multiple genes, including *fgfr2*, *erm*, and *sox9b*, and is essential for the inhibition of neurogenesis. FGFR activation inhibits neurogenesis in part by upregulating the expression of an RA-degrading enzyme, Cyp26b1. We identify *fgf20a* as the critical activator of FGFR essential for the inhibition of neurogenesis, and find that it is expressed by a subset of neurons located at segment centers. These findings reveal a mechanism for the establishment of nonneurogenic zones, in

which FGF signaling from neurons inhibits neurogenesis in the adjacent neural epithelium.

## RESULTS

### Expression of *erm* and *fgfr2* Is Complementary to Neurogenic Zones

Previous studies have revealed that neurogenesis occurs in a dynamic pattern in the zebrafish hindbrain, initially at segment centers, then broadly throughout segments, and later is restricted to zones flanking segment boundaries (Amoyel et al., 2005; Cheng et al., 2004). We further analyzed the restriction of neurogenesis by detection of *neurog1*, *dla*, and *dld* gene expression, which mark the initiation of neurogenesis (Figures 1A–1D and data not shown), and *neurod4*, which is expressed downstream of proneural genes (Roztocil et al., 1997; Wang et al., 2003) (Figures 1E–1H and 1J). We found that the widespread pattern of neurogenesis observed at the 22 somite stage (Figures 1A and 1E) becomes progressively restricted to zones adjacent to hindbrain boundaries, such that, at 36 hours postfertilization (hpf) and 48 hpf, there is an absence of neuronal

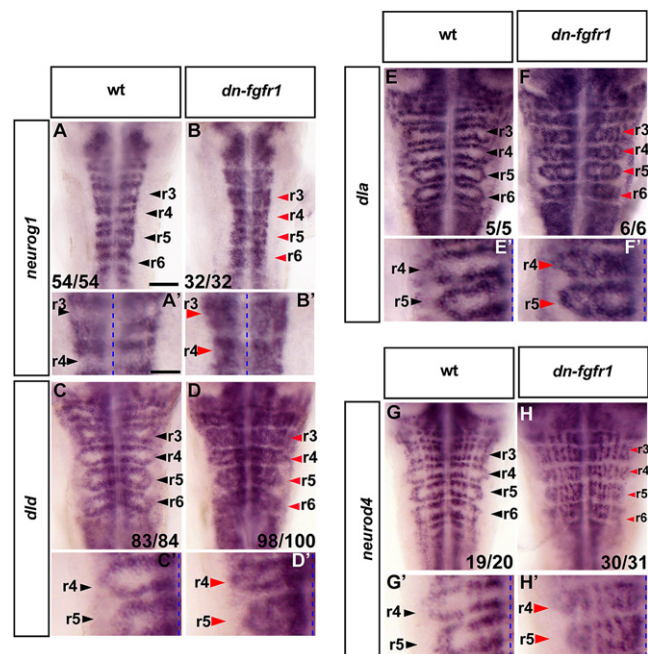
differentiation in the center of rhombomeres (Figures 1C, 1D, and 1G–1J).

These observations raised the question of how neurogenesis becomes restricted during hindbrain development. We obtained clues in searches of publications and the Zebrafish Information Network database (Thisse et al., 2004) for genes that have restricted expression within hindbrain segments. Among these genes, expression of two transcriptional targets of FGF signaling, *erm* and *etv5*, occurs within hindbrain segments at the 20–25 somite and later stages (Munchberg et al., 1999; Roussigne and Blader, 2006). We further analyzed *erm* expression, and found that this occurs in segment centers from the 22 somite stage to 48 hpf, with the level of expression decreasing and becoming a narrower stripe at late stages (Figures 1K–1N). To analyze the relationship with neurogenic zones, we carried out double fluorescent in situ hybridizations. We found that, at 36 hpf, *erm* is expressed in a complementary domain to *dld*, and thus marks the nonneurogenic zone in segment centers (Figures 1O–1Q). Previous studies of *fgfr* gene expression in zebrafish have shown that, in the hindbrain, *fgfr1* is widely expressed, *fgfr3* and *fgfr4* are restricted to r1, and *fgfr2* becomes upregulated in all segment centers (Tonou-Fujimori et al., 2002). We found that *fgfr2* is progressively upregulated and restricted to segment centers from 24 to 48 hpf (see Figures S2A–S2D available online), and is complementary to *neurog1* in neurogenic zones (Figures 1R–1T). Taken together, these findings suggest that expression of *fgfr2* and activation of the FGF pathway occurs in segment centers during the period that neurogenesis becomes restricted to zones adjacent to hindbrain boundaries. We therefore tested the possibility that FGF signaling is involved in the repression of neurogenesis in the hindbrain.

### FGFR Signaling Inhibits Neurogenesis in Segment Centers

Since *fgfs* have been implicated in early stages of hindbrain patterning (Maves et al., 2002; Walshe et al., 2002), analysis of a potential later role requires use of a strategy that allows temporal control of FGFR inhibition or activation. To block FGFR activation, we used a transgenic heat shock-inducible dominant-negative approach (Lee et al., 2005). A fluorescent signal due to expression of dn-FGFR1:EGFP fusion protein can be detected as early as 1 hr after heat shock of *Tg(hsp70l:dnfgfr1-EGFP)* embryos, and persists for at least 24 hr (data not shown). We found that, following heat shock induction of dn-FGFR1 at the 22 somite stage, there is a rapid and persistent decrease in *erm* expression (data not shown). It was important to ascertain that inhibition of FGFR at late stages does not phenocopy the loss of Fgf3/Fgf8, which are transiently expressed in rhombomere 4 and required for r5/r6 segmentation and correct formation of early-born reticulospinal neurons (Maves et al., 2002; Walshe et al., 2002). We found that induction of dn-FGFR1 at the 22 somite stage led to no change in segmental expression of EphA4 in r3/r5 or in formation of reticulospinal neurons (Figures S1A–S1D).

To test whether blocking FGFR activation affects neurogenesis, we analyzed markers of the initiation and later steps of neuronal differentiation. To inhibit FGFR activation, we induced dn-FGFR1 expression by heat shock of 22 somite embryos for 30 min and then incubated for 24 hr before fixation. We found



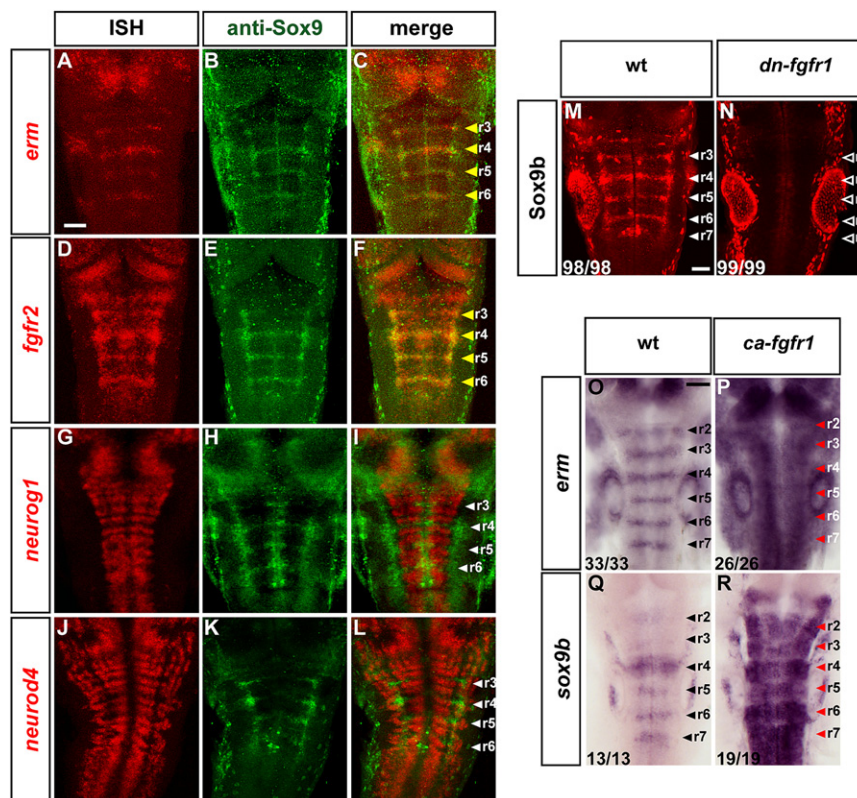
**Figure 2. Blocking FGFR Activation Results in Proneural Gene Expression and Differentiating Neurons in Segment Centers**

In situ hybridization of 40 hpf zebrafish to detect proneural gene expression (*neurog1*, *dld*, *dla*; [A–F] and [A'–F']) or differentiating neurons (*neurod4*; [G and H and G' and H']) in either wild-type (wt) or dominant-negative *fgfr1* embryos (*Tg(hsp70l:dnfgfr1-EGFP)*). Heat shock was started at the 22 somite stage. Black arrowheads indicate segment centers; red arrowheads indicate ectopic neurogenesis. (A–H) Scale bar, 50 μm. (A'–H') Higher-power view of images in (A)–(H); scale bar, 25 μm. Dashed line, midline. See also Figure S1.

that this led to ectopic expression of *neurog1*, *dld*, *dla*, and *neurod4* in the center of segments (Figures 2A–2H and 2A'–2H'). In contrast, there was no ectopic expression in hindbrain boundaries, consistent with previous studies that have implicated Notch activation in the inhibition of neurogenesis (Cheng et al., 2004). Interestingly, not all cells in segment centers upregulate proneural genes following FGFR inhibition, but, rather, neurogenesis occurs in a punctate manner in longitudinal columns coextensive with the normal sites of differentiation in neurogenic zones (Figures 2G'–2H'). These observations suggest that the ectopic differentiation in segment centers is subject to dorso-ventral patterning and lateral inhibition of neurogenesis as occurs in the normal neurogenic zones. Similar phenotypes were observed following treatment of embryos with the FGFR inhibitor, SU5402 (Figures S1E–S1H, S1G', and S1H'). Since FGFR2 is upregulated in segment centers, we carried out morpholino oligonucleotide-mediated knockdowns to analyze whether it is required for the inhibition of neurogenesis. We did not detect any change in neurogenesis (data not shown), suggesting that other FGFRs mediate sufficient levels of FGF signaling.

Since FGF signaling can promote the proliferation of neural progenitors, we analyzed whether blocking with dn-FGFR1 or inducible expression of constitutively active FGFR1 (Marques et al., 2008) affects cell proliferation in the hindbrain. We found that there was no significant change in the number of





**Figure 3. FGF Signaling Maintains a Sox9b-Expressing Population in Segment Centers**

(A–L) In situ hybridization for *erm*, *fgfr2*, *neurog1*, or *neurod4* (red), followed by immunostaining with anti-Sox9 antibody (green). Images shown are a projection of confocal stacks. Scale bar, 50  $\mu$ m. White arrowheads indicate segment centers; yellow arrowheads indicate colocalization of Sox9b with *fgfr2* and *erm*. The staining in segment centers is due to Sox9b, as it is lost in Sox9b morphant embryos (not shown).

(M and N) Whole-mount immunostaining of Sox9b in 36 hpf wt (M) or transgenic dominant-negative *fgfr1* embryos (*Tg(hsp70:dnfgfr1-EGFP)*) (N). White arrowheads indicate segment centers in wt embryos; open arrowheads in transgenic embryos point at centers where Sox9b expression is absent. Images shown are merged confocal stacks. Scale bar, 20  $\mu$ m.

(O–R) In situ hybridizations of 26 hpf wt embryos (left) or embryos expressing constitutively active FGFR1 (*Tg(hsp70:ca-fgfr1)*), using *erm* (O and P) or *sox9b* (Q and R) probes. Embryos were heat shocked at 24 hpf and fixed 2 hr later. Arrowheads indicate upregulation of *erm* or *sox9b* expression. Scale bar, 50  $\mu$ m. See also Figure S2.

pH3-expressing cells after blocking endogenous FGFR activation, or increased FGFR1 activation (data not shown). The inhibition of neurogenesis by FGFR activation in segment centers is thus not associated with a role in the promotion of cell proliferation.

#### FGFR Signaling Regulates Segment Center Restricted Gene Expression

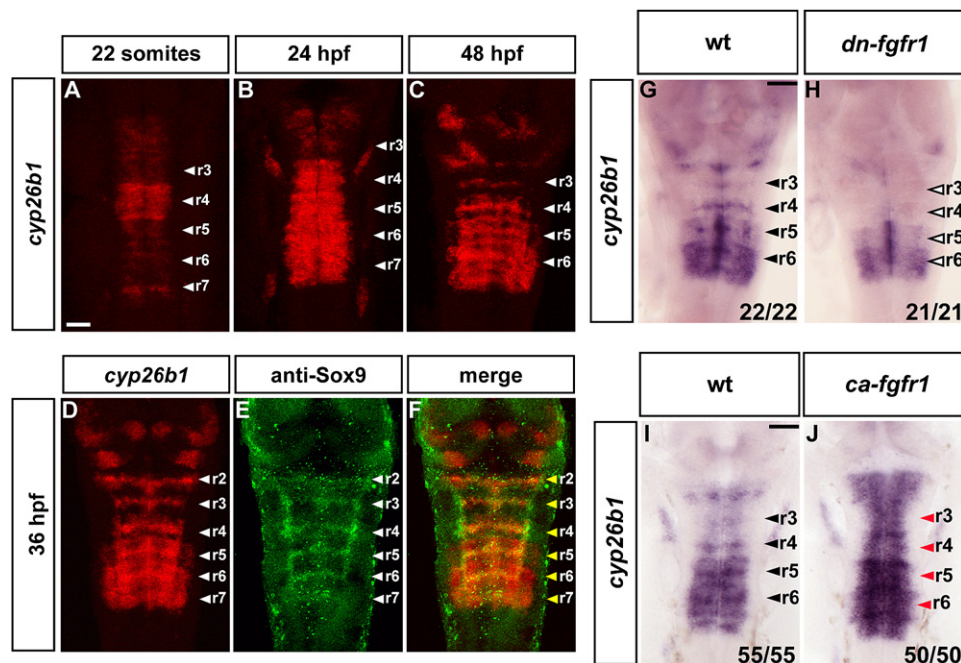
Our results raise the question of whether the inhibition of neurogenesis by FGFR signaling involves formation of progenitors in segment centers that have a distinct specification from those within neurogenic zones. Previous studies have shown that, in other tissues, Sox9 is upregulated by FGF signaling (Govindarajan and Overbeek, 2006; Murakami et al., 2000; Schmahl et al., 2004), and, in the nervous system, Sox9 has been implicated in glial cell differentiation (Stolt et al., 2003). It was therefore intriguing that *sox9b* is expressed in a stripe within hindbrain segments (Yan et al., 2005). We found that expression of Sox9b protein colocalizes with *erm* and *fgfr2* mRNA in segment centers, and is excluded from neurogenic zones (Figures 3A–3L). These data raise the possibility that Sox9b is expressed downstream of FGFR signaling in the hindbrain. To test this, we first analyzed the effect of transgenic expression of dn-FGFR1, and found that Sox9b expression is absent when FGFR signaling is blocked (Figures 3M and 3N). In order to check that the decrease in Sox9b expression is not due to a general loss of neural progenitors, we analyzed *sox3* expression, which marks progenitors throughout the nervous system (Pevny and Placzek, 2005; Uwanogho et al., 1995). We found that inhibition of FGFR signaling at these stages has no detectable effect on *sox3* expression (Figures S2I and S2J).

Since another possible explanation of decreased Sox9b expression is that it is secondary to loss of the expressing cell population, we tested the effect of expressing constitutively activated FGFR1. We analyzed expression of known targets of FGFR activation, and found that there is a high level of *erm* expression throughout the embryo 2 hr after induction of activated FGFR1 (Figures 3O and 3P). Transgenic embryos with overactivation of FGFR1 have a major increase in *sox9b* levels, mostly in areas within the nervous system where it is normally expressed (Figures 3Q and 3R).

The observation that *fgfr2* expression occurs in segment centers (Figures 1R–1T) (Tonou-Fujimori et al., 2002) raised the possibility that this gene is upregulated by the FGF pathway. In agreement with this, we found that *fgfr2* expression was abolished following induction of *dn-fgfr1* expression (Figures S2E and S2F), and that transgenic expression of activated *fgfr1* leads to widespread expression of *fgfr2* (Figures S2G and S2H). Taken together, these results identify a distinct population of neural progenitors in the center of hindbrain segments, in which *erm*, *sox9b*, and *fgfr2* are expressed downstream of FGFR activation.

#### Cyp26b1 Is a Target of FGFR Signaling in the Hindbrain

A clue to a further signaling pathway that may regulate neurogenesis in the hindbrain came from the observation that *cyp26b1* is expressed in segment centers (Gu et al., 2005; Reijntjes et al., 2007; Zhao et al., 2005). Cyp26b1 belongs to the Cyp26 family of proteins, which catabolize RA (Fujii et al., 1997; White et al., 1996; White and Schilling, 2008). Since RA induces neuronal differentiation in different contexts (Maden, 2007), an enzyme that degrades RA constitutes a good candidate to be required



**Figure 4. *Cyp26b1* Is Expressed in Segment Centers and Regulated by FGF Signaling**

(A–D) In situ hybridizations to detect the time course of *cyp26b1* expression. Images are a merge of confocal stacks of Fast Red staining. Scale bar, 50  $\mu$ m. White arrowheads indicate segment centers. (D–F) In situ hybridization of *cyp26b1* followed by immunostaining with anti-Sox9b antibody. Yellow arrowheads show colocalization of *cyp26b1* with Sox9b in segment centers.

(G and H) In situ hybridization of 40 hpf embryos to detect *cyp26b1* expression in wt (G) or dominant-negative FGFR1 embryos (*Tg(hsp70l:dnfgfr1-EGFP)*) (H). Heat shocks were started at the 22 somite stage. Black arrowheads indicate segment centers; open arrowheads indicate the disappearance of *cyp26b1* expression from centers. Scale bar, 50  $\mu$ m.

(I and J) In situ hybridization of 26 hpf embryos to detect *cyp26b1* in either wt (I) or constitutively active *fgfr1* embryos, *Tg(hsp70l:ca-fgfr1)* (J). Heat shocks were started at 24 hpf and embryos fixed 2 hr later. Black arrowheads indicate segment centers; red arrowheads centers in embryos with *cyp26b1* upregulation. Scale bar, 50  $\mu$ m.

for the inhibition of neurogenesis. We therefore analyzed *cyp26b1* expression in more detail, and found that it occurs in a complex and dynamic pattern in the hindbrain between the 22 somite stage to 48 hpf (Figures 4A–4F). *Cyp26b1* expression occurs throughout this period in segment centers, overlapping with Sox9b expression (Figures 4D–4F), as well as at lower levels in some hindbrain boundaries. In addition, *cyp26b1* is expressed more broadly throughout segments in a dynamic pattern in the posterior hindbrain (Figures 4B–4D), which, at 36 hpf, occurs in a graded manner in r5 and r6.

The expression of *cyp26b1* in segment centers prompted us to analyze whether this was dependent upon FGFR activation. We found that expression of dn-FGFR1 led to loss of *cyp26b1* expression in segment centers, whereas segmental expression in r5 and r6 was not affected (Figures 4G and 4H). Conversely, expression of constitutively active FGFR1 led to upregulation of *cyp26b1* in the hindbrain 2 hr after induction of the transgene (Figures 4I and 4J). *cyp26b1* is therefore upregulated downstream of FGFR signaling in segment centers.

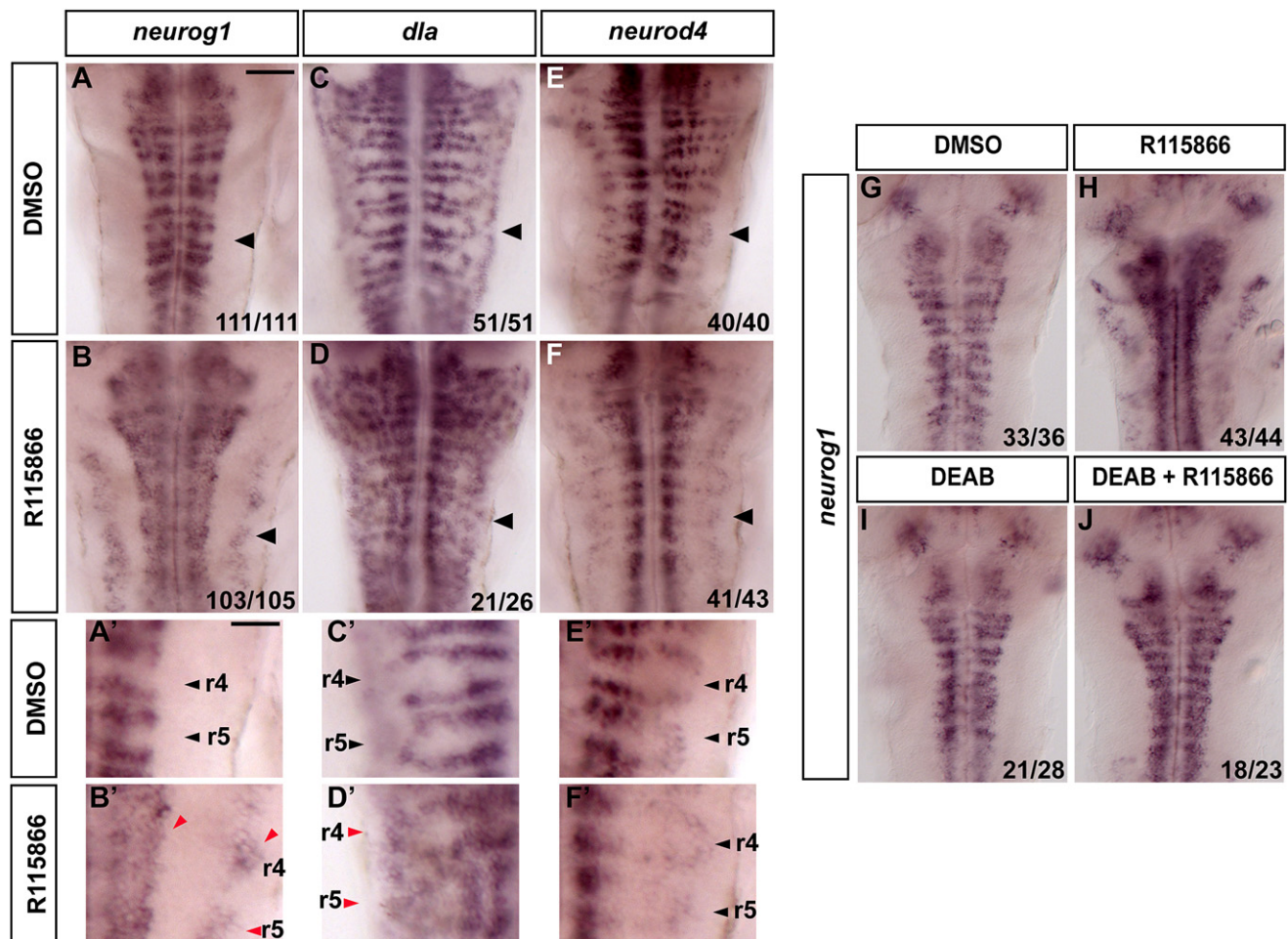
#### Loss of Cyp26 Activity Results in Ectopic Initiation of Neurogenesis

The expression pattern of *cyp26b1* and its regulation by the FGFR pathway raised the possibility that catabolism of RA is

required for the inhibition of neurogenesis in segment centers. Since Cyp26 family members have essential, early roles in anteroposterior patterning of the hindbrain (Hernandez et al., 2007; Uehara et al., 2007; White et al., 2007), we took a pharmacological approach by using R115866, which is a specific inhibitor of Cyp26 enzymes that results in increased RA signaling in vivo (Stoppie et al., 2000). We found that treatment of embryos with R115866 from 24 to 40 hpf led to ectopic expression of *neurog1* and *dla* in segment centers (Figures 5A–5D). However, expression of *neurod4* was not upregulated in segment centers following inhibition of Cyp26 activity during this period (Figures 5E and 5F).

These results suggest that inhibition of Cyp26 activity leads to the ectopic upregulation of proneural gene expression that initiates neurogenesis, but is not sufficient for the progression of neuronal differentiation marked by *neurod4*. This is in contrast to the effect of FGFR inhibition, which leads to ectopic expression of both proneural markers and *neurod4* in segment centers (Figure 2). We therefore analyzed whether blocking of Cyp26 activity affects expression of segment center markers. We found that, in most embryos treated with R115866, *fgfr2* (54% of embryos;  $n = 68$ ), *cyp26b1* (72%;  $n = 25$ ), and Sox9b (84%;  $n = 44$ ) are still expressed in segment centers. These findings suggest that expression of Cyp26 contributes to inhibition of





**Figure 5. Blocking Cyp26 Activity Results in Premature Neurogenesis**

(A–F) In situ hybridization of 40 hpf embryos to detect expression of *neurog1* (A and B), *dla* (C and D) or *neurod4* (E and F) in DMSO- or R115866-treated embryos. Treatments were started at 24–26 hr. Black arrowhead points at r5. Scale bar, 50  $\mu$ m. (A'–F') Higher-power views of r4 and r5 shown in A–F (black arrowheads). Red arrowheads indicate ectopic proneural expression. Scale bar, 25  $\mu$ m.

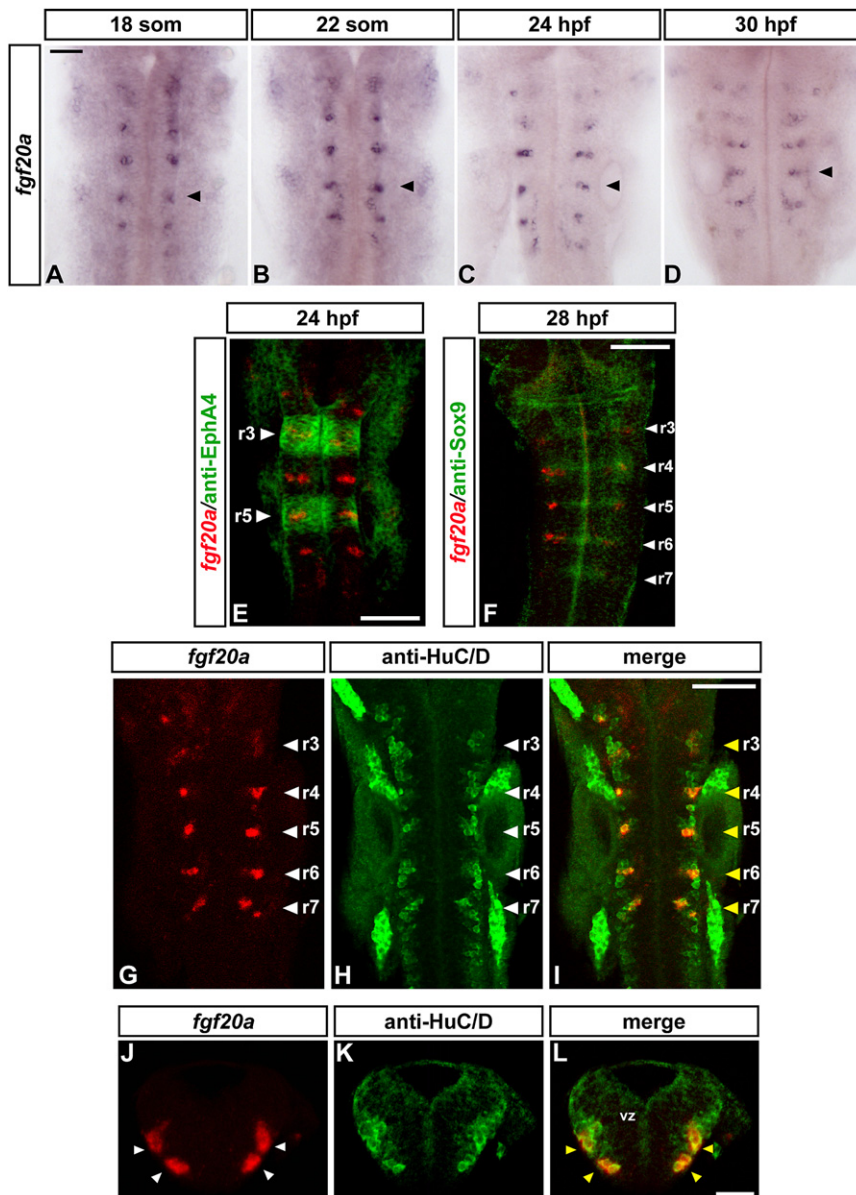
(G–J) Blocking RA signaling with DEAB partially rescues loss of Cyp26. In situ hybridization of 36 hpf embryos to detect expression of *neurog1* in DMSO (G), R115866 (H), DEAB (I), or R115866 + DEAB (J) -treated embryos.

the initiation of neurogenesis, but FGFR signaling also induces other genes that regulate different aspects of maintaining an undifferentiated population.

The finding that excess RA due to *cyp26* inhibition leads to ectopic neurogenesis raises the question of whether retinoid signaling is required for neurogenesis within the normal neurogenic zones. We found that treatment of embryos with DEAB to block the RA synthesis enzyme, RALDH2, has no effect on the expression of neurogenic markers (Figures 5G and 5I). To determine whether DEAB does affect RA signaling in the context of hindbrain neurogenesis, we tested whether cotreatment with DEAB rescues the effect of excess RA due to blocking of Cyp26 enzymes. We found that the ectopic neurogenesis that occurs following Cyp26 inhibition is suppressed by cotreatment with DEAB (Figures 5H and 5J). These results reveal that the level of RA affects neurogenesis in segment centers, whereas RA is not essential for neurogenesis adjacent to hindbrain boundaries.

#### ***fgf20a* Is Expressed by Neurons at Segment Centers**

Our findings raise the important questions of the identity and site of expression of the FGF(s) that activate FGFRs in segment centers. We therefore carried out in situ hybridization analyses of zebrafish FGF genes to determine their expression pattern during the relevant period of hindbrain development. This identified *fgf20a* as a potential candidate. *fgf20a* starts to be expressed in the hindbrain at the 14 somite stage in a few isolated cells (data not shown) and, by the 18 somite stage, is detected in a cluster of cells in each of rhombomeres 2–7 (Figure 6A). Similar *fgf20a* expression in discrete cell populations is observed at least until 36 hpf (Figures 6B–6D; Figures S3J–S3L), and is undetectable by 48 hpf (data not shown). The *fgf20a*-expressing cells are located at segment centers (Figure 6E), at the same antero-posterior location as Sox9b expression (Figure 6F). To determine whether *fgf20a* expression occurs in progenitors or neurons, we carried out double staining with the panneuronal marker, HuC/D,



**Figure 6. *fgf20a* Is Expressed in Neurons at Segment Centers**

(A–D) Time course of *fgf20a* expression at 18 somites (A), 22 somites (B), 24 hpf (C), and 30 hpf (D). Embryos belong to the same batch and were developed for the same amount of time. Black arrowheads point at the center of r5. Scale bar, 50 μm. (E–I) Merge of confocal stacks of double-stained embryos. Scale bar, 100 μm. (E) In situ hybridization of *fgf20a* (red) and anti-EphA4 staining (green) to reveal r3 and r5 (white arrowheads) at 24 hr.

(F) In situ hybridization of *fgf20a* (red) and antibody staining for Sox9b (green) in 28 hpf embryo. White arrowheads indicate segment centers.

(G–I) In situ hybridization of *fgf20a* (red) and antibody staining for the panneuronal marker HuC/D in 24 hpf embryos. White arrowheads indicate segment centers; yellow arrowheads show colocalization of *fgf20a* with specific HuC/D-expressing neurons.

(J–L) Double labeling of *fgf20a* (red) and HuC/D (green). Images show a merge of confocal stacks through r4 at 24 hpf in transverse sections (dorsal is to the top). In r4, *fgf20a*-expressing cells form clusters (white arrowheads) in the mantle zone and colocalize with specific HuC/D-expressing neurons (yellow arrowheads). VZ, ventricular zone. Scale bar, 50 μm. See also Figure S3.

and analyzed transverse sections. We found that *fgf20a*-expressing cells are located in the mantle zone and correspond to a subset of neurons (Figures 6G–6I; Figures S3A–S3I). The location of these neurons at segment centers suggests that *fgf20a* is a good candidate for restricting neurogenesis in the hindbrain.

#### ***fgf20a* Maintains Segment Center Markers and Restricts Neurogenesis**

To analyze the role of *fgf20a*, we analyzed mutants that harbor a temperature-sensitive null allele of the *fgf20a* gene (Whitehead et al., 2005). We found that *fgf20a* mutants have normal segmental expression of ephrinB3 (data not shown), and thus appear not to be required for the early role of Fgf signaling in r5/r6 segmentation. In contrast, there is a major decrease in *erm* expression in segment centers in 24 hpf *fgf20a* mutants,

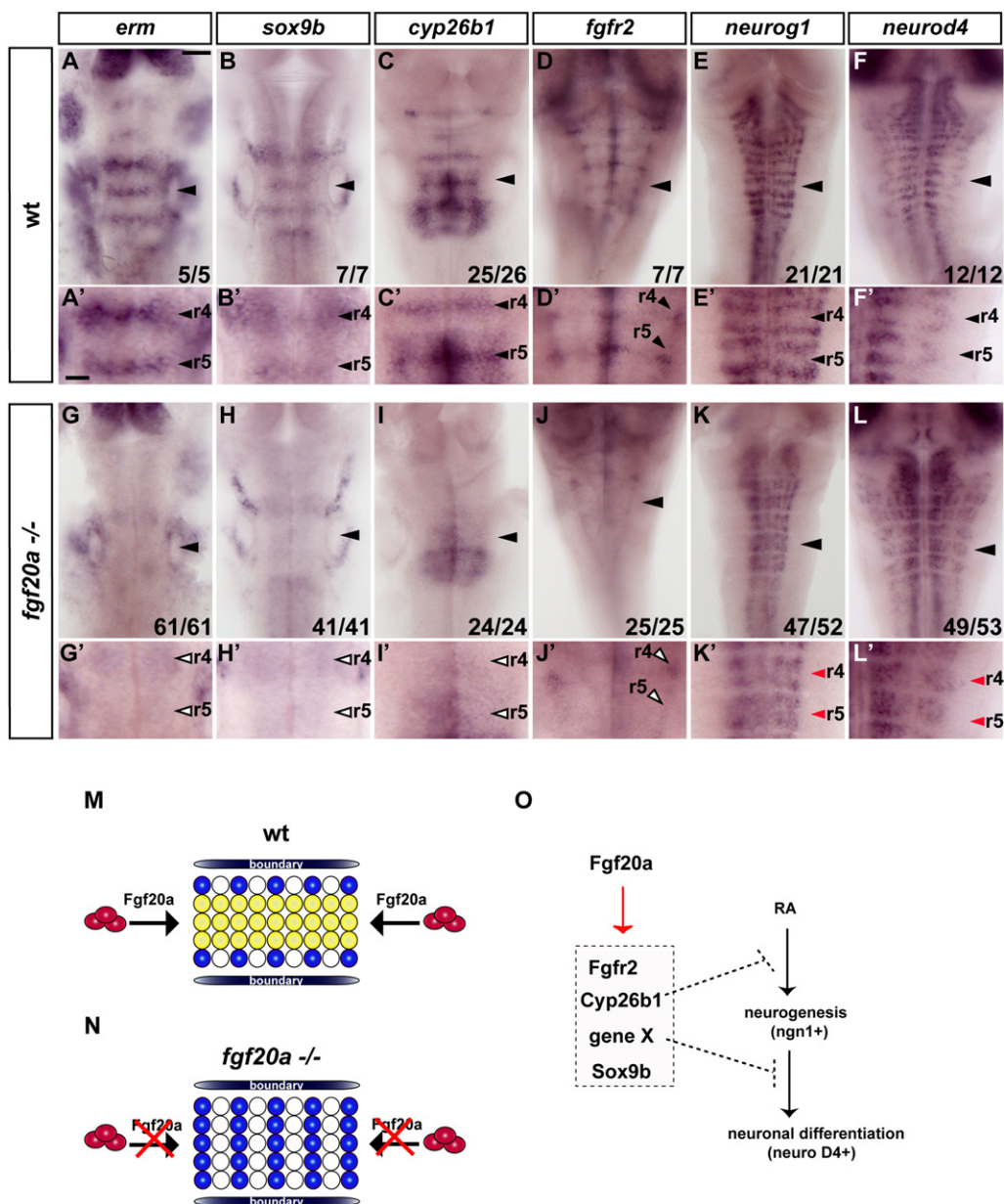
whereas expression in other tissues, such as the midbrain-hindbrain boundary, appears to be at normal levels (Figures 7A, 7A', 7G, and 7G'). Similarly, there is decreased expression of *sox9b*, *cyp26b1*, and *fgfr2* (Figures 7B–7D, 7B'–7D', 7H–7J, and 7H'–7J') in segment centers in *fgf20a* mutants.

These findings reveal that *fgf20a* is required for the FGFR-dependent maintenance of segment center markers. We therefore analyzed whether loss of *fgf20a* function affects neurogenesis in the hindbrain. We found that, in 40 hpf *fgf20a* mutants, there is ectopic expression of *neurog1* and *neurod4* in segment centers (Figures 7E, 7E', 7F, 7F', 7K, 7K', 7L, and 7L'), as occurs following the blocking of FGFR activation by expression of *dn-fgfr1* or by SU5402 treatment. Taken together, our data show that *fgf20a*, which is expressed by early-born neurons located at segment centers, has a crucial role in maintaining segment center markers and in spatial restriction of neurogenesis in the hindbrain.

#### **DISCUSSION**

Generation of the appropriate number and type of neural cell types requires precise regulation of cell differentiation and maintenance of progenitors. In some regions of the nervous system, this involves formation of spatially segregated neurogenic and nonneurogenic regions that are induced downstream of axial





**Figure 7. *fgf20a* Is Required for Inhibition of Neurogenesis in Segment Centers**

(A–L) In situ hybridizations of wt (A–F) or *fgf20a* homozygous embryos (G–L) raised at 25°C. A'–L' show higher-power images of A–L. Scale bar, 50  $\mu$ m for A–L; 20  $\mu$ m for A'–L'. *erm* expression in segment centers is significantly reduced in *fgf20a* mutants (open arrowheads in G'). Markers of segment centers, *sox9b*, *cyp26b1*, and *fgfr2*, are greatly decreased in *fgf20a*<sup>-/-</sup> embryos (open arrowheads [H'–J']). (K and L) *fgf20a* mutant embryos have ectopic neurogenesis in segment centers, detected by *neurog1* (E and K) and *neurod4* expression (L and F). Red arrowheads indicate ectopic neurogenesis in segment centers (K' and L').

(M and N) Model of the patterning of neurogenesis by *fgf20a* in hindbrain segments. In wt embryos (M), *fgf20a* secreted from neurons in the adjacent mantle region (red ovals) prevents neuronal differentiation (blue circles) in segment centers by maintaining a population of progenitors (yellow circles). (N) In *fgf20a* mutants, there is ectopic neurogenesis and low-level expression of segment center markers.

(O) Summary of the regulation of genes in the nonneurogenic zone of progenitors in segment centers. *fgf20a* upregulates a set of genes that control different aspects of maintaining an undifferentiated population.

patterning mechanisms or by localized inhibitory signals within the neural epithelium (Bae et al., 2005; Bally-Cuif and Hamerschmidt, 2003; Bertrand et al., 2002; Brewster et al., 1998; Kawauchi et al., 2005; Saarimaki-Vire et al., 2007). In the zebrafish hindbrain, neurogenesis becomes restricted to zones

adjacent to segment boundaries, and is absent in segment centers and boundaries. Previous studies suggest that Notch activation underlies the inhibition of neurogenesis at segment boundaries (Cheng et al., 2004). Here, we show that the non-neurogenic zone in segment centers is marked by the expression



of a number of genes, including *fgfr2*, *erm*, *sox9b*, and *cyp26b1*. Overactivation of FGFR leads to upregulation of segment center markers within 2 hr, suggesting that they are early targets of FGF signaling, whereas blocking FGFR activation leads to downregulation of these markers. Furthermore, inhibition of FGFR leads to ectopic neurogenesis in segment centers in longitudinal columns coextensive with the normal pattern of neuronal differentiation adjacent to boundaries. We identify *fgf20a* as the critical activator of FGFR in segment centers. *fgf20a* is expressed by a subset of neurons located in the mantle layer at the center of segments, and, in the *fgf20a* mutant, there is a spreading of neurogenesis and downregulation of segment-center marker expression. These studies have uncovered a mechanism in which signaling from specific early-born neurons underlies formation of a nonneurogenic zone (Figures 7M and 7N).

### Roles of FGF in the Inhibition of Neurogenesis

Previous studies have found diverse roles of FGF signaling in the regulation of neurogenesis. In some contexts, specific FGFs promote neuronal differentiation; for example, FGF15 in the mouse cerebral cortex (Borello et al., 2008) and FGF8 in the early *Xenopus* forebrain (Hardcastle et al., 2000). However, a more common role of FGF signaling is to promote the proliferation of neural epithelial cells and maintain progenitors required for subsequent differentiation to postmitotic neurons. For example, a null mutation in FGF2 leads to a reduced number of neurons in the mouse neocortex (Ortega et al., 1998) and cortex (Vaccarino et al., 1999), consistent with a role in maintaining the neural stem-cell pool (Zheng et al., 2004). Our findings suggest that, in hindbrain segment centers, FGF signaling maintains a distinct progenitor zone by inhibiting neuronal differentiation, but is not required for the promotion of cell proliferation. Similarly, the sites of FGF pathway activation in the adult zebrafish brain correspond to radial glial cells, some of which may act as neural progenitors, but do not correlate with cell proliferation (Topp et al., 2008).

Roles of FGF signaling sources in local inhibition of neurogenesis occur in other regions of the developing nervous system. For example, FGFs expressed at the midhindbrain boundary act through multiple FGFR family members in the mouse to maintain a zone of progenitors (Jukkola et al., 2006; Saarimäki-Vire et al., 2007). Similarly, in the olfactory epithelium, localized expression of FGF8 in the nasal pit maintains an adjacent zone of proliferating progenitor cells, with neuronal differentiation occurring distal from the FGF source (Kawauchi et al., 2005). Our findings are suggestive of an analogous role of *fgf20a* in the local inhibition of neurogenesis in segment centers in the hindbrain. Whereas, in these other examples, an FGF source within the neural epithelium underlies the local inhibition of neurogenesis, in the hindbrain it is due to an FGF expressed by early-born neurons.

### Antagonism between FGF and RA Signaling in Neurogenesis

Previous work has shown that RA has an important role in the promotion of neurogenesis in a number of regions of the nervous system (Maden, 2007). Studies of the caudal spinal cord in chick embryos have revealed an antagonistic relationship between RA and FGF signaling in the control of neuronal differentiation

(Diez del Corral et al., 2003). FGF8 expression in caudal regions represses expression of RALDH2 required for synthesis of RA, whereas RA attenuates FGF8 expression. Consequently, there are counter gradients of FGF and RA activity, such that neurogenesis is inhibited in caudal regions where there is high FGF and low RA signaling, and initiated more rostrally where there is low FGF and high RA. By analogy, it is possible that the expression of the RA-catabolizing enzyme, *cyp26b1*, in hindbrain segment centers is required for the inhibition of neurogenesis downstream of FGFR activation. Consistent with this, we found that *cyp26b1* expression in segment centers requires FGFR activation, and that inhibition of Cyp26 proteins leads to ectopic expression of proneural and Delta genes that mark the initiation of neurogenesis. However, in contrast to the effect of blocking FGFR activation, Cyp26 inhibition did not lead to expression of *neurod4*, which marks a later step of neuronal differentiation. Furthermore, expression of *fgfr2* and *Sox9b* was maintained following Cyp26 inhibition, and, thus, the expression of these genes is regulated by FGFR independently of inhibition of RA signaling. Based upon these findings, we propose that FGFR activation in segment centers upregulates multiple genes that regulate different aspects of neurogenesis (Figure 7O): decreased RA signaling due to expression of *Cyp26b1* contributes to inhibition of the onset of neurogenesis in segment centers, but unidentified targets inhibit subsequent steps of neuronal differentiation.

Intriguingly, inhibition of RA synthesis by DEAB does not affect neurogenesis in the normal neurogenic zones adjacent to boundaries, yet DEAB suppresses ectopic neurogenesis in segment centers that occurs following inhibition of Cyp enzyme activity. RA is therefore not essential for neurogenesis in the hindbrain, but increased RA is sufficient to drive initiation of neurogenesis, and Cyp26 enzymes are required to prevent this in segment centers. The simplest explanation of these findings is that another neurogenic factor(s) acts in parallel with RA, and is present at a sufficient level adjacent to hindbrain boundaries.

### Significance of Signaling from Neurons

Our findings suggest that the formation of nonneurogenic zones is due to feedback inhibition in which the generation of *fgf20a*-expressing neurons limits subsequent neurogenesis. Feedback inhibition mediated by other signals has been found to limit the amount of neurogenesis. In the case of lateral inhibition by Notch ligands expressed by nascent neurons, this acts at short range within neurogenic zones, and occurs transiently, since it is relieved once the differentiating neuron has migrated into the mantle layer. A more sustained feedback inhibition occurs in the olfactory epithelium. The BMP family member, GDF11, is expressed by differentiating progenitors and olfactory receptor neurons downstream of the proneural gene, *mash1*, and acts to limit the amount of further neurogenesis and maintain progenitors (Wu et al., 2003). GDF11-expressing nascent neurons are distributed widely in the neural epithelium and adjacent mantle layer, and do not mediate a spatial patterning of neurogenesis.

Our studies raise the question of whether formation of a nonneurogenic zone in segment centers has roles other than in limiting the amount of neurogenesis. One model is that some cells in segment centers migrate into the neurogenic zones and thus provide a supply of progenitors for subsequent

differentiation distal from the FGF source, as may occur in the nasal epithelium (Kawauchi et al., 2005). Similarly, cells in the nonneurogenic zone at the midhindbrain boundary in zebrafish later contribute to neurogenesis throughout the adjacent region (Tallafuss and Bally-Cuif, 2003). Another non-mutually exclusive possibility is that the upregulation of Sox9 expression in hind-brain segment centers underlies a switch from neuronal to glial cell differentiation, as found in the mouse spinal cord (Stolt et al., 2003). One role of the nonneurogenic zone may therefore be to enable generation of glial cell types.

### FGF20 and Homeostasis

The generation and maintenance of the correct number of progenitors and differentiated derivatives is crucial during both development and tissue homeostasis in the adult. It is intriguing that FGF20, which is expressed in the substantia nigra, has been implicated in the survival of dopaminergic neurons (Murase and McKay, 2006; Ohmachi et al., 2000), a role that may underlie the reported association between FGF20 haplotypes and Parkinson's disease (van der Walt et al., 2004). It will be interesting to determine whether FGF20 also contributes to maintenance of progenitors in the adult nervous system, analogous to the developmental role that we have uncovered. Roles of FGF20 in tissue homeostasis have been revealed in studies of the zebrafish fin. The normal regeneration of the fin following injury does not occur in the *fgf20a* mutant, due to a requirement for *fgf20a* in formation of the blastema that generates the missing differentiated tissues (Whitehead et al., 2005). Furthermore, *fgf20a* is required for the homeostatic maintenance of tissue during cell turnover in uninjured fins (Wills et al., 2008). Taken together with our findings, these studies raise the interesting possibility that FGF20 acts in diverse tissues to maintain progenitor cells by participating in feedback loops that underlie homeostasis.

### EXPERIMENTAL PROCEDURES

#### Zebrafish Strains, Husbandry, and Genotyping

Zebrafish embryos were staged according to hpf and morphological criteria (Kimmel et al., 1995). *fgf20a* (dob [devoid of blastema]) mutant embryos (Whitehead et al., 2005) were obtained from homozygous *fgf20a* incrosses and raised at 25°C from 3 hpf until fixation. Transgenic embryos *Tg(hsp70l:dnfgr1-EGFP)* and *Tg(hsp70:ca-fgr1)* used for this work are heterozygotes from outcrosses. *Tg(hsp70l:dnfgr1-EGFP)* and *Tg(hsp70:ca-fgr1)* adult carriers were identified as previously described (Lee et al., 2005; Marques et al., 2008). *Tg(hsp70l:dnfgr1-EGFP)* embryos were identified after heat shock by the fluorescence of the *fgr1*-EGFP fusion protein. To confirm that phenotypes observed in embryos derived from *Tg(hsp70:ca-fgr1)* zebrafish correlated with presence of the transgene, individual embryos were genotyped after in situ hybridization. Briefly, after photographing embryos mounted in glycerol, they were washed overnight in PBT at 4°C, incubated in 50  $\mu$ l TE for 10 min at 98°C, and then overnight at 55°C in TE containing 200  $\mu$ g/ml proteinase K. After inactivation for 10 min at 98°C, genomic DNA was used for PCR amplification to detect Dsred transgene with the primers, CATCTGTCCCCCAGTTCC and CCCAGCCCATAGTCTTCTTCTGC.

#### Heat Shock and Pharmacological Treatments

To inducibly block FGFR activation, *Tg(hsp70l:dnfgr1-EGFP)* embryos and wild-type littermates were heat shocked for 30 min at 38.5°C. Treatments were started at the 22 somite stage, and embryos fixed 24 hr later. To induce constitutively active FGFR by using *Tg(hsp70:ca-fgr1)*, 24 hpf embryos were heat shocked for 30 min at 38.5°C, transferred for 2 hr at 28.5°C, and then fixed. To pharmacologically block FGFR, 22 somite embryos were incubated

for 24 hr in 100  $\mu$ M SU5402 (Calbiochem) or an equivalent dilution of DMSO carrier as control. To block Cyp26 activity, 24–26 hpf zebrafish embryos were treated with 50  $\mu$ M R115866 (Janssen Pharmaceutica) for 24 hr, or with equivalent dilutions of DMSO as control. To block RA signaling, 50  $\mu$ M DEAB was added to 1–3 somite embryos. To block Cyp26 activity in DEAB-treated embryos, R115866 was added at 50  $\mu$ M at 8 somites, and embryos fixed at 36 hr. Embryos were in their chorions for all drug treatments.

#### In Situ Hybridization

Whole-mount in situ hybridization was carried out as previously described (Xu and Wilkinson, 1998), with the following modifications. Embryos were prehybridized in hybridization mix (50% formamide, 5 $\times$  SSC [pH 4.5], 50  $\mu$ g/ml yeast RNA, 100  $\mu$ g/ml heparin, 0.2% Tween20, 5 mM EDTA) for 2 hr at 68°C. Hybridization was carried out at 68°C overnight, then the following washes were carried out at 68°C: 5 min in 66% formamide/33% 2 $\times$  SSC; 5 min in 33% formamide/66% 2 $\times$  SSC, 5 min in 2 $\times$  SSC, 0.1% Tween20; 15 min in 0.2 $\times$  SSC, 0.1% Tween20; and twice for 15 min in 0.1 $\times$  SSC, 0.1% Tween20. Final washes at room temperature were for 5 min each in 66% 0.1 $\times$  SSC, 33% PBT, then 33% 0.1 $\times$  SSC, 66% PBT, and then 100% PBT. For digoxigenin detection, embryos were blocked in 5% sheep serum and then incubated with anti-digoxigenin-AP antibody (Roche) (1:1500 dilution) at 4°C overnight. Finally, embryos were washed all day and overnight in PBT at room temperature and color developed with either NBT/BCIP (Roche) or Fast Red (Roche). Double fluorescent in situ hybridization was performed as previously described (Julich et al., 2005), followed by detection with tyramide signal amplification (tyramide labeled with Alexa Fluor 488 or 594) following the manufacturer's instructions (Molecular Probes). The following probes were used: *erm* (cb805), *sox9b* (MGC:76805), *fgr2* (gift of Ivor Mason), *neurog1*, *neurod4*, *dla*, and *dld* (Amoyel et al., 2005; Cheng et al., 2004), *cyp26b1* (Hernandez et al., 2007), *fgf20a* (Whitehead et al., 2005), and *sox3* (IMAGE clone ID: 3726393). Photographs were taken with confocal microscopy (Leica TCS SP2).

#### Whole-Mount Immunofluorescence

For whole-mount antibody staining, embryos were fixed for 2 hr at room temperature in 4% paraformaldehyde, rinsed in PBT, dechorionated, and blocked for 1 hr in 5% goat serum in PBT. Embryos were then incubated overnight at 4°C in the required antibody at the following dilutions: rabbit anti-Sox9 (1:500 [Morais da Silva et al., 1996]; gift from Silvana Guilioli), rabbit anti-EphA4 (1:450 [Irving et al., 1996]), mouse anti-HuCD (1:100; Molecular Probes), mouse anti-neurofilament (1:25, Zymed), in 2.5% goat serum. Secondary goat antibodies used were Alexa Fluor conjugates (Invitrogen).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.devcel.2009.11.010.

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